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REVIEW ARTICLE

Effects of exogenous amines on mammalian cells, with particular reference to membrane flow

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Introduction

Amines are the molecules which result from the substitution of one, two or all of the hydrogens of ammonia, NH_3, with organic radicals (these comprise, respectively, primary, secondary, and tertiary amines). They are important mammalian biosynthetic products, as neurotransmitters (e.g. noradrenaline) and intercellular mediators (e.g. histamine), and intracellularly (e.g. cystamine: Freedman & Hawkins, 1980). Several cell types (e.g. platelets, adrenal medulla cells) store amines such as serotonin or noradrenaline at very high concentrations in specialized intracellular vesicles (Apps, 1982). Ammonia itself (mainly from transamination and deamination, and notably in the kidney) and other amines are also significant catabolites. However, the concentration of ammonia in extracellular fluids (with the occasional exception of urine) is very low (normally less than 1 mM). Cultured cells can generate ammonia, but normally the resultant extracellular concentrations are again low.

Many widely used drugs are amines; perhaps the most notable three categories being the tertiary amine local anaesthetics (e.g. lidocaine) and calcium blockers (e.g. verapamil), the phenothiazine tranquillizers, and the antimalarial (and anti-inflammatory) chloroquine and its congeners. The actions of the anaesthetics and calcium blockers involve complex effects on monovalent and divalent cation channels (e.g. Lee & Tsien, 1983) and calcium binding sites on membranes which are not shared with all amines, and thus are not detailed here. The mechanisms of amine antimalarial function are not understood (Peters, 1980). However the archetype of this group of drugs, chloroquine, has been widely used experimentally, and many of the effects discussed below may be relevant to antimalarial action. Amines are also in widespread use as buffers (e.g. Tris), components of hair-dyes and of cigarettes (Cohen & Roe, 1981); and many are carcinogenic (Radomski, 1979; Ashby *et al.*, 1983). Several antibiotics (e.g. the aminoglycosides such as gentamycin: Aubert-Tulkens *et al.*, 1979) which are widely used in cell culture experiments are also amines.

Most amines are weak bases and this is responsible for a characteristic central theme in the ensuing discussion: accumulation in acidic areas of the cell. Many of the effects of amines to be described here would therefore probably be shared by most weak bases, providing their p*K*s are suitable and provided they can enter cells. But it is notable that the vast majority of weak bases listed in a standard work on organic bases (Perrin, 1965) are amines. The exceptions have p*K* or solubility properties that render them unsuitable for use in cellular systems.

This Review concentrates on the effects of exogenously supplied amines. It only considers endogenous amines when they illuminate effects of exogenous amines. Thus the Review is little concerned with several receptors for transmitters, serotonin or histamine (e.g. Arrang *et al.*, 1983), or with the toxicology of amines. Another massive literature concerns the roles of polyamines (such as spermine) and their oxidized derivatives in the control of cell growth (reviewed by Pegg & McCann, 1982). Again this is not discussed here. Granted these exclusions, the main topics of this Review are the intravesicular effects of amines; the related actions on processes involving interactions between different membranes, such as flow of macromolecules between different cellular compartments (both after endocytosis and biosynthesis); and the role of membrane-bound receptors in the latter events. We will consider these actions first by analysing the entry and accumulation of amines in cells. Then we will consider the direct effects of the amines on intracellular compartments and their contents. Subsequently we will deal successively with effects on interactions and transport of receptors and their ligands; and on membrane fusion, with reference to both endocytosis and exocytosis. Fig. 1 summarizes some of the components of endocytic events which may be perturbed by amines, while Fig. 2 does so for exocytosis. The references cited have often been chosen, of neces-

sity, to provide convenient leads to current literature rather than to indicate priority.

Entry and intracellular accumulation of exogenous amines

Weak bases have been known for many decades to cause vacuolation in cells, a gross expansion of the intracellular vesicular system. This is due to their accumulation within cells in regions of low pH. The mechanisms involved in this have been elegantly analysed by de Duve *et al.* (1974). In summary, weak bases penetrate cells in their uncharged form, providing they are sufficiently lipophilic, and rapidly load the cytosol to a concentration of the order of the extracellular concentration (but depending on their p*K*a). Then more slowly they accumulate within acidic organelles (notably lysosomes) to much higher concentrations because they become trapped in the hardly permeable protonated form. The maximum concentration ratio that can be achieved is approximately the ratio of intravesicular to extracellular proton concentration. The rate of accumulation depends also on p*K*, so that compounds of very high p*K*s (approx. 10 or above) take many hours or days to equilibrate, while those with lower p*K*s do so in minutes. Several features of this analysis have since been fully substantiated. For instance, Ohkuma & Poole (1981) have listed many weak bases whose vacuolating capacity they have studied. As predicted, lipophilic weak bases accumulate more rapidly than do hydrophilic ones, and the extent of vacuolation is proportional to the concentration of uncharged base (Poole & Ohkuma, 1981). Uptake involves an energy-independent component, and an active one in which proton pumping by lysosomes seems to be responsible for continued uptake (Ohkuma *et al.*, 1982). Complementary data describing uptake of amines by hepatocytes have recently been published by Solheim & Seglen (1983).

Apps (1982) has reviewed the mechanisms of the related proton-translocating ATPase involved in catecholamine storage in adrenal medulla secretory vesicles; in this case an electrogenic catecholamine carrier also participates. Such coupled amine-carriers may also be present in lysosomes, but have not yet been described. Coated vesicles have recently been shown also to possess an ATP-dependent proton pump (Forgac *et al.*, 1983).

It should also be noted that charged amine species such as the ammonium ion can be carried by surface transporters into cells, with varying degrees of efficiency (Kleiner, 1981; Stefaniu & Charandini, 1982). Furthermore, several cation ionophores are potent in carrying charged species of amines into cells (e.g. X537A: Kinsel *et al.*, 1982).

Sites of intracellular accumulation of amines

Direct evidence has been supplied by many authors that, as anticipated, lysosomes are a major site of accumulation (Wibo & Poole, 1974; Poole & Ohkuma, 1981). However, they are not the only site; the former authors also noted that chloroquine causes 'peripheral dilatation of golgi cisternae', and Posner *et al.* (1981) have found that chloroquine is accumulated there. Chloroquine-induced autophagic vacuoles in rat liver also accumulate chloroquine (Gray *et al.*, 1981). Most recently evidence that several amines perturb the pH of endosomes (Helenius & Marsh, 1982) and phagosomes (Young *et al.*, 1981) indicates that these are further sites of accumulation.

Effects on the pH of intracellular compartments

Ammonia and some other amines are acidotic with respect to the extracellular fluid *in vivo*, but they cause elevation of the pH of acidic intracellular compartments. Ohkuma & Poole (1978) presented an impressive method for measuring the pH of such compartments, using the pH-dependence of fluorescence of fluorescein-labelled dextran. They showed (Poole & Ohkuma, 1981) that changes in the pH of media had little effect on macrophage intralysosomal pH, but that amines rapidly elevate lysosomal pH in a concentration-dependent manner, while vacuolation with other agents such as sucrose had no such effect. The normal lysosomal pH of about 4.8 could be raised at most to about 6.2. Tycko & Maxfield (1982), using fluorescein-conjugated α_2-macroglobulin in a similar way, have shown that pinocytic vesicles also become acid very rapidly, almost certainly prior to fusion with lysosomes. However Young *et al.* (1981) revealed that phagosomes undergo a fairly brief alkalinization before they become acid, and even this process is inhibited by NH_4Cl, though not by chloroquine. Both these amines inhibit the subsequent acidification, as might be expected. The difference between the effects of the two amines may result from the somewhat slower penetration of chloroquine than ammonium. Though Poole & Ohkuma (1981) showed that the pH change considerably anticipates the completion of uptake of the amines into the lysosomal compartment, so that the later accumulation must be due to progressive neutralization of buffering capacity (whose extent is unclear), or subsequent proton pumping, or both. In contrast, Reijngoud & Tager (1977) maintain that the pH shifts produced in fibroblasts by amines are due to simple neutralization of protons by the weak bases. Hollemans *et al.* (1981), from the same group, have demonstrated the acidity of lysosomes in human fibroblasts, and the accumulation of weak bases

therein; but find a smaller pH rise due to amines than do Poole & Ohkuma (1981). While the mechanism is thus still not entirely clear, there is general agreement that the pH of several intracellular compartments rises in the presence of amines; the evidence is direct in the case of certain kinds of phagosome and pinosome, and of lysosomes. Some cationic ionophores, notably monensin, can also destroy the pH gradient across lysosomal and other membranes (Tycko & Maxfield, 1982).

Distribution and metabolism in the organism of exogenously supplied amines

When supplied to intact organisms, much of the dose of an amine is excreted (Cohen & Roe, 1981; Radomski, 1979). This has been much studied for the aminoglycoside antibiotics because of their nephrotoxic properties (Riff & Jackson, 1971). Detailed reviews on the distribution within organs of circulating amines are available (e.g. Gillis & Pitt, 1982). Significant quantities remain intracellularly, as discussed above. In addition, some molecules may undergo further reactions, although the quantitative significance of these pathways is unclear.

Most of these pathways are not relevant to the purpose of the present Review and thus are not discussed here. Some pathways lead to incorporation into proteins: for instance, peroxidation (Thomas *et al.*, 1982). This may be relevant to some of the effects of amines on membranes, as may incorporation due to transglutaminases (Clarke *et al.*, 1959; Butter & Landon, 1981).

Direct effects of amines on enzymes

Primary amines inhibit the action of transglutaminases on their experimental substrates (Leu *et al.*, 1982). This has been used to attempt to establish the role of that enzyme in certain receptor-mediated processes (see below). A related capacity is that of penicillamine to react with the aldehyde intermediate in the cross-linking of lysines in collagen, thereby preventing cross-linking (Freedman & Hawkins, 1980).

The induction of phospholipidosis in cells *in vitro* and *in vivo* (particularly in kidney) by aminoglycosides and other amines (Lullmann-Rauch, 1981) has led to the study of direct effects of amines on enzymes of lipid catabolism independent of effects on pH. Both phospholipases A_1 and A_2 in cellular extracts can be inhibited by aminoglycosides and chloroquine (Laurent *et al.*, 1982; Hostetler & Richman, 1982). In addition, sphingomyelinase in rat fibroblast extracts is inhibited by gentamycin (Aubert-Tulkens *et al.*, 1979). The mechanism of these inhibitions is not clear; besides direct effects on the enzymes, inhibition may involve changes in the substrates due to their complexing with the amines (Seeman, 1972; Laurent *et al.*, 1982; Lullman & Vollmer, 1982). Wibo & Poole (1974) reported that chloroquine at 100mM can also inhibit cathepsin B; concentrations of that order seem to be attained in lysosomes. Finally, high concentrations of chloroquine can also inhibit α-galactosidase (de Groot *et al.*, 1981).

Immediate consequences within vesicles of amine accumulation and pH elevation

The complete degradation of macromolecules within lysosomes depends on the maintenance within those organelles of an acidic pH, since it is fulfilled by enzymes with acid pH optima (Dean & Barrett, 1976). Thus when cells are exposed to sub-toxic amine concentrations (approx. 10mM-NH_4Cl or 10μM-chloroquine), these processes are inhibited. Some well-established examples are listed in Table 1. Since bulk proteolysis is a determinant of growth, amines might in some circumstances enhance cellular growth by reducing proteolysis. However, the limited evidence available does not support this idea (e.g. King *et al.*, 1981). Besides these gross catabolic events, limited proteolytic steps may also depend on vesicles of low pH (for instance, removal of pro-pieces of secretory and some other proteins, such as lysosomal enzymes: reviewed by Sabatini *et al.*, 1982). Inhibition of such limited proteolysis may contribute to the inhibition of antigen presentation by amines (Julian *et al.*, 1983; Chesnut *et al.*, 1982; Grey *et al.*, 1982). A recent example in which such inhibition of limited proteolysis may obtain is in the maturation of adrenocorticotropin from its precursors in a mouse pituitary cell line (Moore *et al.*, 1983; Dean, 1983). This possibility merits direct study.

In many of these cases it has been argued that evidence of inhibition of the catabolic step(s) by amines that disrupt pH gradients of acidic compartments indicates that the step(s) are performed by lysosomes or related acidic organelles. While there is direct evidence *in vitro* that degradation of proteins in isolated lysosomes can be inhibited when chloroquine is added (Ahlberg *et al.*, 1982), this does not necessarily prove the case. In particular, indirect effects, such as those consequent on an inhibition of protein synthesis (see below), must be considered. For instance, protein degradation in cells is regulated by availability of amino acids, which increases if protein synthesis is retarded (Hershko & Ciechanover, 1982); a consequent diminution of degradation could be expected. Further, the indications of changed flux of vesicles (below), and the evidence that Golgi vesicles are

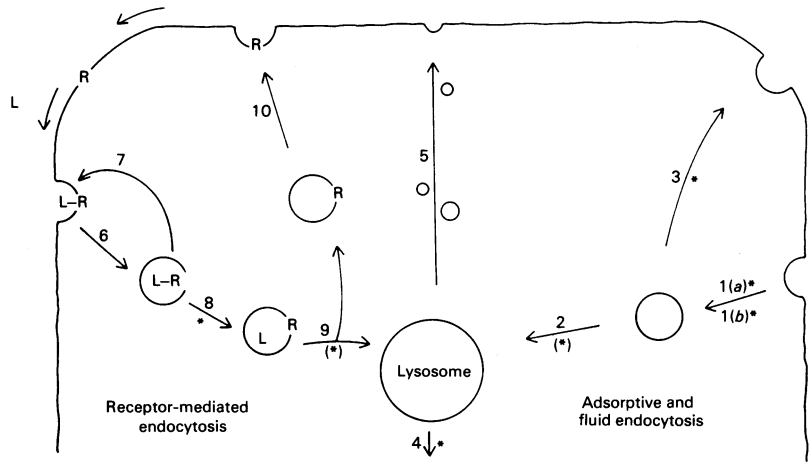

Fig. 1. *Effects of amines on endocytic pathways*

* Indicates points at which amines have been shown to have direct effects; (*) indicates points at which amines may have effects, but for which the evidence is as yet incomplete. Note that each arrow represents a segment of a pathway that may comprise several discrete steps. Symbols and key: L, ligand; R, receptor; 1(*a*), internalizaton of fluid and adsorbed materials; 1(*b*), acidification of endosome; 2, endosome–lysosome fusion; 3, retro-endocytosis and membrane recycling; 4, lysosomal degradation; 5, membrane recycling; 6, internalization of receptor–ligand complexes; 7, recycling to plasma membrane of receptor–ligand complexes; 8, acidification of endosome and dissociation of receptor–ligand complexes; 9, delivery of ligand to lysosomes; 10, recycling of receptor to plasma membrane.

Table 1. *Inhibition of degradation by amines*

As discussed in the text, the inhibition of degradation of the specific proteins mentioned here may partly be due to prevention of their access to the degradative compartment, rather than to direct inhibition of degradation. The Table is highly selective, in order to serve as a convenient entry to the literature.

Substrate	References
Bulk protein of long half-life	Seglen *et al.* (1979)
In basal degradation	Amenta & Brocher (1980)
In nutritional restriction	Seglen & Gordon (1979)
	see Dean (1980)
In confluent culture	Cockle & Dean (1982)
Specific proteins	
Endogenous	
Membrane proteins (e.g. acetylcholine receptor)	Libby *et al.* (1980)
Newly synthesized secretory proteins	Berg *et al.* (1980)
Newly synthesized glycosaminoglycans	Lie & Schofield (1973)
Endocytosed proteins	
Adsorptively pinocytosed	Livesey *et al.* (1980)
Pinocytosed via specific receptors	
Epidermal growth factor	King *et al.* (1981)
α_2-Macroglobulin	Kaplan & Keogh (1981)
Cholesterol esters	
Cholesterol linoleate	Brown *et al.* (1978)

also sites of accumulation of amines (Posner *et al.*, 1981), reveal that diversion of substrate independent of (as well as dependent on) retarded degradation in an acidic compartment might be involved.

There are now well-documented examples of alteration of intracellular translocation due to such elevation of vesicular pH. The archetype is the exit of Semliki Forest virus from an acidic intracellular environment (probably an endosome) into the cytosol in which it can replicate (Helenius & Marsh, 1982). This escape mechanism is inhibited by amines. Passage of virus through a membrane can occur at the cell surface if extracellular pH is artificially low. A similar mechanism has been demonstrated for other viruses, and it may apply to several protein toxins which penetrate membranes to damage cells (Houslay & Elliott, 1981; Sandvig & Olsnes, 1982), and whose effects are inhibited by

amines. This mechanism may be relevant to other proteins.

Modification of protein synthesis by amines and other cytosolic effects

In general, concentrations of NH_4Cl up to 10mM, and of chloroquine up to 20μM, have insignificant effects on protein synthesis in cultured cells (Amenta & Brocher, 1980; Jessup *et al.*, 1983). In many cells, higher concentrations are very inhibitory, but these effects, at least in the case of hepatocytes, can be overcome by addition of metabolites (e.g. amino acids: Seglen, 1978). As discussed below, the availability or output of several proteins (thromboplastin, plasminogen activator, viral membrane protein) is enhanced by amines to such a degree that it is possible that their synthesis is increased.

It has long been argued that changes in cytosolic pH are crucial in growth control and differentiation (see Nuccitelli & Deamer, 1982). Such controlling roles would necessarily involve modification of protein turnover and protein expression. Since amines raise the pH of the cytosol a little, it is not surprising that in certain circumstances both growth and differentiation can be modified by amines (for a recent, non-mammalian, example see Gross *et al.*, 1983). However, it is not known which intracellular compartments are important in such circumstances.

Effects of amines on receptor–ligand dissociation

Receptors that probably function intracellularly

Fibroblasts can endocytose exogenously supplied lysosomal enzymes by means of a receptor specific for mannose 6-phosphate ligands on the enzyme (Kaplan *et al.*, 1977*a*,*b*; Ullrich *et al.*, 1978; Sando *et al.*, 1979; Gonzalez-Noriega *et al.*, 1980). Although this receptor is now thought to function intracellularly in transport of newly synthesized lysosomal enzymes, this understanding has depended much on studies of endocytosis (Sly *et al.*, 1981). Under normal conditions the endocytosed receptor–ligand complexes are thought to dissociate when they enter a region of low pH, such as lysosomes or endosomes.

Incubation of cells with extracellular lysosomal enzymes in the presence of amines causes a reduction in the rate of receptor-mediated endocytosis due to a decrease in the number of mannose 6-phosphate receptors available at the cell surface (Wiesmann *et al.*, 1975; Sando *et al.*, 1979; Gonzalez-Noriega *et al.*, 1980). The mechanism by which receptor availability is depressed is not known, but it has been suggested that amines by raising the pH of relevant compartments may prevent dissociation of internalized complexes, resulting in a progressive intracellular accumulation of occupied receptors. However, no direct demonstration of this has been made; it is not clear why the occupied receptors should be any less capable of returning to the plasma membrane than are the unoccupied ones. Thus it is possible that occupied receptors accumulate also at the cell surface. A major effect of this putative depletion of free mannose 6-phosphate receptors in fibroblasts incubated with amines is that newly synthesized lysosomal enzymes are immediately exported from the cell (Gonzalez-Noriega *et al.*, 1980; Hasilik & Neufeld, 1980*a*,*b*), presumably because their normal segregation into lysosomes (rather than to secretion) depends on their binding to the unoccupied receptors in the Golgi/endoplasmic reticulum. Amine treatment depresses the availability of receptors at that site also. Sly *et al.* (1981) consider that mannose 6-phosphate receptor-mediated pinocytosis of extracellular enzyme is secondary to the intracellular function of the receptor. The macrophage cell line $P388D_1$ secretes its lysosomal enzymes spontaneously (Jessup & Dean, 1980), and they contain the mannose 6-phosphate ligand (Jessup & Dean, 1982; Gabel *et al.*, 1982). This is apparently due to a deficiency of the receptor (Gabel *et al.*, 1983). As discussed below, amines depress this spontaneous secretion (Jessup *et al.*, 1982). It is interesting that Sahagian & Gottesman (1982) find that the major secreted protein of transformed mouse fibroblasts carries the mannose 6-phosphate markers, though it is not particularly well endocytosed. This protein seems to be lysosomal, and its output from normal cells is stimulated by NH_4Cl and monensin; output from transformed cells is unaffected by similar concentrations of these compounds.

Such an intracellular pH-dependent sorting mechanism may apply more widely than to lysosomal protein alone. For instance, since chloroquine perturbs secretion of adrenocorticotropin forms, Moore *et al.* (1983) propose that one may be at work in this system too. Other explanations are plausible however (Dean, 1983; see below).

It should be noted that alteration of flux of proteins along intracellular translocation pathways does not necessarily entail any change in flux of vesicles themselves (see below), since the content of the vesicles alone might change.

Receptors that probably function at the plasma membrane

Several receptor-mediated endocytic systems that operate as routes for the high-affinity uptake of extracellular macromolecules are sensitive to amines (Table 2). Incubation of cells with some

Table 2. *Inhibition of receptor recycling by amines*

This Table lists only the most extensively documented examples of inhibition of receptor recycling by amines; even in these systems there is still need for interpretive caution, because of the indirect nature of the available evidence.

Receptor	Cell type	Amines	Reference
Mannose-glycoprotein termini	Rat alveolar macrophage	Ammonium, chloroquine	Tietze *et al.* (1980, 1982)
Asialoglycoprotein	Rat hepatocyte	Chloroquine, dansylcadaverine; dibucaine	Tolleshaug & Berg (1979); Tolleshaug *et al.* (1982)
α_2-Macroglobulin–proteinase complexes	Human fibroblast	Methylamine, dansylcadaverine	Van Leuven *et al.* (1980)
	Rabbit alveolar macrophage	Chloroquine, methylamine	Kaplan & Keogh (1981)
Low density lipoprotein	Human fibroblast	Chloroquine	Basu *et al.* (1981)
Epidermal growth factor	Human fibroblast	Methylamine, chloroquine	King *et al.* (1980)
Mannose 6-phosphate termini	Human fibroblast	Ammonium, chloroquine	Gonzalez-Noriega *et al.* (1980)

amines produces a reduction in the net rate of receptor-mediated ligand uptake.

In early studies a correlation between the ability of primary amines such as methylamine and dansylcadaverine to prevent ligand uptake and to inhibit transglutaminase (Davies *et al.*, 1980; Maxfield *et al.*, 1979*a*,*b*; Levitzki *et al.*, 1980) led to the suggestion that this enzyme was essential in the internalization process. However, subsequent studies demonstrated that (*a*) several inhibitors of transglutaminase, such as bacitracin, do not inhibit ligand uptake (Haigler *et al.*, 1980; King *et al.*, 1981) and (*b*) amines do not prevent receptor-mediated internalization of ligands such as mannose-glycoproteins (Tietze *et al.*, 1980) or epidermal growth factor (King *et al.*, 1980). Rather amines reduce net ligand uptake by reducing the number of unoccupied receptors at the cell surface. This depletion is rapidly reversed when amines are withdrawn (Tietze *et al.*, 1980), which suggests that it is due to accumulation of receptors within an intracellular pool, rather than to accelerated receptor degradation. The mechanism by which amines prevent receptor recycling is not known; it may be direct or through changes in membrane flux (see below).

That recycling of several types of receptor (mannose-glycoprotein: Tietze *et al.*, 1980; epidermal growth factor: King *et al.*, 1980; α_2-macroglobulin: Kaplan & Keogh, 1981; low density lipoprotein: Anderson *et al.*, 1977) is prevented by amines in the absence of extracellular ligand suggests that this inhibition does not result simply from accumulation of occupied receptor–ligand complexes within intracellular vesicles, due to the elevation of their pH (as has been proposed for the mannose 6-phosphate system). However, in several instances, simultaneous presentation of ligand and amine considerably enhances the inhibitory effect of amines on receptor recycling (Kaplan & Keogh, 1981; Tietze *et al.*, 1980, 1982). Thus this mechanism may also significantly contribute to amine-induced inhibition of receptor recycling.

An interesting contrast is provided by the uptake of the iron-carrier protein transferrin by cells bearing its receptor (reviewed by Octave *et al.*, 1983). While amines depress uptake of transferrin, little emphasis has been placed on the study of receptor recycling and its inhibition. Rather, it has been shown that dissociation of iron from transferrin intracellularly only occurs in a low pH region, and is blocked by amines. The receptor and ligand remain associated at acid or the more neutral pH caused by amines within the cell, and a significant proportion recycles undissociated so that apotransferrin is made available again extracellularly. In view of the apparent normal behaviour of the receptor–ligand complex intracellularly in this system even when perturbed by amines, it will be particularly interesting to establish the nature of the inhibition of ligand uptake they cause.

In summary, inhibition of receptor recycling probably involves several components including the inhibition of return of unoccupied intracellular receptors to the cell surface, and prevention of the regeneration of free receptors from internalized receptor–ligand complexes. Fig. 1 summarizes some of the sites of action of amines which are relevant to this section. The central difficulty in the published studies is to achieve unambiguous labelling of the intracellular receptors. This is highlighted by the current debate as to the number of such receptors present even in such a relatively well characterized system as the hepatocyte asialoglycoprotein receptor (e.g. Bridges *et al.*, 1982).

Effects of amines on membrane structure and interactions

Direct studies on membrane fusion and other modifications of membranes exerted by amines

Tertiary amine anaesthetics modify the extent of membrane fusion (Poste & Allison, 1973; Lucy, 1982). This has been mainly revealed by studies of fusion of cells and of liposomes. In contrast, there has been rather less work which directly demonstrates their effects on fusion of intracellular membranes either *in vivo* or *in vitro*. The idea that such agents inhibit fusion of fluid membranes by displacing Ca^{2+} from membrane sites is still under assessment. Indeed, this property is shared by several aminoglycoside antibiotics (Lullmann & Vollmer, 1982). However, as noted above, many of the documented features of tertiary amine local anaesthetics may not be shared by lysosomotropic amines generally. Thus the degree of applicability of these ideas to the membrane flow processes discussed next is far from clear.

Almost as widely studied as the tertiary amine local anaesthetics are the anti-inflammatory chloroquine (also a tertiary amine, and effective as a local anaesthetic: Mandel, 1960), and the phenothiazine tranquillizers (reviewed by Weissmann, 1969). Both groups were viewed as acting directly on membranes. Thus they can stabilize lysosomal membranes against various physical insults *in vitro*. In many cases the effects show biphasic concentration-dependence (Weissmann, 1969). In most of these cases insufficient work has been done to decide whether alterations in fusion also result. But chloroquine in particular not only produces vacuolation of cells, but also increases numbers of autophagic vacuoles containing detectable materials such as membrane whorls, etc. (Fedorko *et al.*, 1968). This may reflect solely the inhibition of catabolism described already; or it may additionally involve altered membrane fusion. Other membrane actions of amines such as displacement of calcium may be involved in their inhibition of hepatocyte spreading (Seglen & Gordon, 1979).

One function of surface calcium that might aid membrane fusion is in the catalysis of cross-links between membrane proteins by endogenous membrane transglutaminases (Lorand *et al.*, 1976). Histamine inhibits this process (Lorand *et al.*, 1976). But Schindler *et al.* (1980) report that polyamines decrease the mobility of membrane proteins of the erythrocyte, which is normally consequent on cross-linking. Nevertheless there is some evidence that histamine and cystamine can inhibit erythrocyte membrane fusion induced by uranyl acetate and rare earth metals (Majumdar *et al.*, 1980), while Kent (1982) found that lysosomotropic amines do inhibit myoblast fusion. However, histamine and dansylcadaverine do not prevent myoblast fusion, although they do inhibit myotube formation (Bersten *et al.*, 1983). The latter amines may act on intracellular transglutaminases involved in myotube formation subsequent to myoblast fusion. These superficially conflicting data indicate that interpretation of membrane fusion effects solely in terms of protein mobility changes is unlikely to be sufficient.

The only other recent direct study of effects on non-anaesthetic amines on membrane fusion concerns the fusion of host cells induced by certain strains of Herpes Simplex virus (Kousoulas *et al.*, 1982). NH_4Cl (50mM) inhibits cell fusion; at the same time the cell surface virally coded glycoproteins seem to be modified in their glycosylation, but remain capable of inducing fusion. The inhibition of fusion induced by the amine thus may be a direct effect on host cell membrane components.

There has been much interest in the idea that primary amines acting on transglutaminases prevent clustering of molecules on the cell surface (Davies *et al.*, 1980). But in later, more detailed, studies Yarden *et al.* (1981) showed that while patching of epidermal growth factor on 3T3 cells is prevented for about 15min, clustering does occur after 30–45min. Microclustering (two to six molecules) is unaffected, and endocytosis can occur before patch formation. Conflicting data still appear and systems apparently vary (Dickson *et al.*, 1982). But as mentioned, these alterations of membrane aggregation do not seem essential for endocytosis.

Another study has shown that coated vesicles can be disaggregated *in vitro* by protonated amines though at the gross concentration of 500mM (Keen *et al.*, 1979). Whether this is an action on clathrin itself, on some other external component, or on intravesicular components, is not known. This is in contrast to the indications of Libby *et al.* (1980) that much lower concentrations of amines may retard uncoating of vesicles, and so reduce their fusion and degradation of their contents (see below).

Clearly there is a need for studies of effects of a wide range of amines on membrane fusion *in vitro* and on other membrane properties. Such studies need to address both effects due to external binding of amines and those due to alterations in internal environment of the membranes, such as pH, membrane potential, and protein cross-linking and motility in membranes.

Other studies of plasma membrane function in cell–cell and cell–substrate interactions

A specialized interaction controlled by the plasma membrane is that between several kinds of

cytotoxic cell and their targets, which are often transformed cells. Because such killer cells are often purified from sources contaminated with erythrocytes, which are removed by lysis with 80mM-NH_4Cl, the effects of NH_4Cl on cytotoxicity have been documented (e.g. Savary *et al.*, 1979). Subsequent cytotoxicity mediated by natural killer (NK) cells is reduced by prior exposure to the amine. However, there seems to have been no detailed study of the mechanism responsible. There are considerable data implicating carbohydrate recognition sites, usually either mannose or mannose and fructose phosphates, in the interaction between killers and targets (Muchmore *et al.*, 1980; Stutman *et al.*, 1980). In the light of our discussion of recycling, it might readily be envisaged that the amine treatment of the killer cells depletes their surface of necessary receptors for binding the target. However, without direct information on the effects of amines present during (as opposed to before) cytolysis, many other possibilities (including direct membrane effects) remain eligible.

Effects on intracellular fusion events

Following uptake of extracellular materials by endocytosis, many internalized vesicles normally rapidly fuse with lysosomes giving hydrolases access to their contents. In studies of the degradation of endocytosed asialoglycoproteins by rat hepatocytes, exposure to amines (ammonium, chloroquine, lidocaine, dansylcadaverine, dubucaine) in the interval immediately following endocytosis resulted in the accumulation of undegraded material within the cells (Berg & Tolleshaug, 1980; Tolleshaug *et al.*, 1982). By both differential and density gradient centrifugations, the intact endocytosed proteins were shown to sediment with a vesicle population distinct from lysosomes. These vesicles were presumed to represent endosomes, and thus it was concluded that amines prevent the transfer of internalized material from endocytic vesicles to lysosomes. Merion & Sly (1983) have presented confirmatory data on epidermal growth factor, hexosaminidase and low-density lipoprotein in human fibroblasts, showing that chloroquine and monensin reduce transfer between two intermediate structures and lysosomes. This inhibition may indicate a reduced rate of fusion between these intracellular structures, caused by the amines, but this has not been established.

Asialoglycoproteins and the ligands studied by Merion & Sly (1983) enter the chosen cells predominantly by receptor-mediated endocytosis. There is much less data available on the fate of endocytic vesicles containing non-specifically adsorbed materials. Schneider & Trouet (1981) using cultured fibroblasts found that uptake and degradation of an immunoglobulin G obtained from a serum that was not directed against the cells was substantially retarded by chloroquine and methylamine. But its transfer intracellularly from the plasma membrane to lysosomes seemed unaffected, whereas that of an anti-(plasma membrane) immunoglobulin G was. More detailed studies on this point are needed; furthermore there seems to be no analogous information on the transfer of fluid contents of endocytic vesicles to lysosomes in the presence of amines. As suggested by Kovacs *et al.* (1981), fusion of other substrate-bearing vesicles (such as autophagic vesicles, derived intracellularly) with lysosomes may frequently be inhibited by amines.

D'Arcy Hart and colleagues have observed the effects of amines on phagosome–lysosome fusion in macrophages by pre-labelling secondary lysosomes with Acridine Orange (itself a weak base) and then following microscopically their fusion with yeast-containing phagocytic vesicles. In this system, 10mM-NH_4Cl supplied immediately after incubation of the cells with yeast significantly inhibits subsequent phagosome–lysosome fusion (Gordon *et al.*, 1980) in common with its effect on endocytic vesicle–lysosome fusion. In contrast, several other amines (including chloroquine) enhance fusion (D'Arcy Hart & Young, 1978). Chloroquine also reverses the inhibition of fusion by certain polyanionic macromolecules (Geisow *et al.*, 1980; D'Arcy Hart & Young, 1978). The explanation for the differential effects of the various amines is not clear. However, since these observations were made at few concentrations for each amine, interpretation is difficult. Since dose–response profiles of several amine effects are biphasic, such data would be valuable here.

Vesicle–plasma membrane fusion

We suggested earlier that amines affect the range of materials exported from cells by their influence on the types of molecules which are inserted into forming secretory vesicles. We now discuss evidence that amines can also modify secretion by modifying the rate at which various types of intracellular vesicles fuse with the plasma membrane.

Under normal culture conditions a significant portion of pinocytosed fluid is rapidly re-released by a range of different cell types (Besterman *et al.*, 1981; Adams *et al.*, 1982; Dean & Jessup, 1982). 'Re-cycling' of membrane (with consequent release of fluid) from incoming endosomes or secondary lysosomes to the plasma membrane had already been predicted by Steinman *et al.* (1976) as a homeostatic mechanism for maintenance of a constant cell surface area; the same idea is well known in the context of the neuromuscular junction,

where a burst of secretion of acetylcholine is succeeded by accelerated endocytosis which retrieves membrane from the cell surface. Besterman *et al.* (1983) have suggested that the release of fluid is modulated by physiological levels of amino acids. We observed that several amines have a complex effect on this phenomenon in fibroblasts, causing an inhibition of efflux which is maximal at relatively low (10–20mM-NH_4Cl, 1 μM-chloroquine) and reduced at higher (non-toxic), concentrations (Dean & Jessup, 1982). These data suggest an inhibitory effect of amines at low concentrations overlaid with stimulation of exocytosis at higher ones. In agreement with this, nicotine at 13.2mM was previously shown to stimulate the release of endocytosed soluble marker from mouse macrophages, while at 1.32mM it had no effect relative to controls (Schwartz & Bond, 1972). Previously endocytosed lysosomal enzymes are also exocytosed by fibroblasts, and this process too can be enhanced by chloroquine (Vladutiu, 1982). Such re-release is also probably a component of antigen presentation (Julian *et al.*, 1983), which can be inhibited by amines (Chesnut *et al.*, 1982; Grey *et al.*, 1982). This may again result from modification of vesicle–plasma membrane fusion.

There is also some evidence for an inhibitory effect of some amines on the outward flux of secretory vesicles containing various cell-derived molecules. For example, Seglen & Reith (1977) observed inhibition of output of serum proteins from cultured hepatocytes by NH_4Cl. We have recently shown (Jessup *et al.*, 1983) that the constitutive secretion of lysozyme by macrophages and a macrophage cell line ($P388D_1$) can similarly be decreased by this amine. The spontaneous hypersecretion of lysosomal enzymes by $P338D_1$ cells (Jessup & Dean, 1980) due to lack of the mannose 6-phosphate receptors (Gabel *et al.*, 1982) is strongly inhibited by several amines (Jessup *et al.*, 1982). A similar but less marked effect of NH_4Cl on spontaneous I-cell (mannose 6-phosphate ligand-deficient) lysosomal enzyme secretion has also been demonstrated (Jessup *et al.*, 1983). The interpretation of these data is complicated by possible effects of amines on protein synthesis and/or degradation discussed above. In the case of $P388D_1$ cells, NH_4Cl has no effect on net protein synthesis or total culture lysosomal enzyme levels at concentrations which maximally inhibit release, while in I-cells the effects of amines on secretion precede temporally the inhibition of protein synthesis. Thus the effects of amines on fusion of intracellular vesicles with the plasma membrane seem likely to include a primary component rather than to be entirely secondary. Taken together, the data on constitutive secretion of intracellular products and previously endocytosed materials suggest that amines can inhibit vesicle–plasma membrane fusion.

In contrast to their effects on constitutive secretion, amines initiate secretion of lysosomal enzymes by mononuclear phagocytes (Schwartz *et al.*, 1972; Riches & Stanworth, 1980; Riches *et al.*, 1981; Jessup *et al.*, 1982). This process is distinct from the types of fibroblast lysosomal enzyme secretion described above, in that enzymes are released from a preformed intracellular pool, since release is not affected by inhibition of protein synthesis by cycloheximide (Jessup *et al.*, 1983). A wide range of agents which are not amines can also induce the selective release of lysosomal enzymes from macrophages (Davies & Allison, 1976). The mechanisms by which this secretion is initiated are not yet understood, although it has been shown that NH_4Cl influences the process by a route distinct from that of some other initiators such as zymosan (Jessup *et al.*, 1982; Riches & Stanworth, 1982). The long-known discharge of residual bodies (telolysosomes) from certain cells may in some circumstances be enhanced by chloroquine (Stauber *et al.*, 1981), but it is not clear whether this relates to secretion of lysosomal enzymes as just discussed.

Endocytosis

Consistent with the inhibition of the outward flux of vesicles from cells by amines, a comparable inhibition of formation of vesicles at the plasma membrane exists. Fluid phase pinocytosis in macrophages is partially inhibited by nicotine (Schwartz *et al.*, 1972; Thyberg & Nilsson, 1982), methylamine (Kaplan & Keogh, 1981), and NH_4Cl (Jessup *et al.*, 1983). NH_4Cl also dramatically reduces fluid phase endocytosis by rat yolk sacs (Livesey *et al.*, 1980), and adsorptive pinocytosis of albumin in that system. Both Wiesmann (1974) and Sando *et al.* (1979) found only slight effects of amines on dextran pinocytosis using concentrations that significantly inhibited the mannose 6-phosphate-dependent endocytosis of lysosomal enzymes. However, in each instance only a single concentration of each amine was tested.

There are also reports that amines can inhibit phagocytosis. Many studies (e.g. Fidler *et al.*, 1982) have employed local anaesthetic tertiary amines, but others have shown that this effect is more widespread. For instance, Thyberg & Nilsson (1982) showed that nicotine inhibited the phagocytosis of latex particles by macrophages, while Sun-Sang *et al.* (1983) found inhibition of zymosan uptake during simultaneous exposure of macrophages to NH_4Cl or chloroquine. Several primary amines inhibit receptor-mediated phagocytosis in macrophages (e.g. Leu *et al.*, 1982), but it is not clear

whether transglutaminases are involved, for the reasons discussed above.

Effects on transport of other macromolecules that may depend on exocytosis or shedding

Production of plasminogen activator and thromboplastin by mononuclear phagocytes

Here we need to distinguish secretion, the process of selective release of cellular molecules into the extracellular fluid, from its component terms, exocytosis and shedding. Exocytosis is the variety of secretion in which vesicular transport and fusion with the plasma membrane is responsible for the extracellular release of vesicular contents. Shedding is the process in which macromolecules have a finite stay at the plasma membrane before being released into the extracellular fluid either by outwards blebbing of micelles or vesicles, or by proteolytic separation of the secretory part of a protein from a hydrophobic anchor which remains in the membrane (for review see Black, 1980). It may well be that material which is shed is transported to the cell membrane in vesicles, but this is not entirely clear.

Somewhat unexpectedly we have found that release of plasminogen activator by mouse peritoneal macrophages is substantially enhanced by NH_4Cl (Roberts *et al.*, 1983). The enzyme is present on the plasma membrane (e.g. Lemaire *et al.*, 1983). There are no data on the effects of amines on cellular levels of the enzyme, so the nature of the stimulation of release is obscure. Black (1980) has argued that the enzyme is shed rather than exocytosed, but the data are not compelling.

The presence of plasminogen activator at the cell surface makes it analogous to the cellular procoagulant, thromboplastin (Prydz & Lyberg, 1981) which is active there, and normally not released by cells (except by transformed cells: Black, 1980). Pursuing this analogy, we have found that a wide range of amines vastly enhance its production by human monocytes (Dean & Prydz, 1983). Similar but less dramatic effects are produced by the exposure of WISH amnion cells to chloroquine (Maynard *et al.*, 1976). In the monocyte studies, low amine concentrations can be inhibitory, and there are preliminary indications that intracellular transport may be retarded, even when the total cellular concentration of thromboplastin has been elevated.

The increased availability of both plasminogen activator and thromboplastin may result from perturbations of the membrane flow which is required for their transport, the route of which is unknown. Alternatively, a sorting system which depends on a low pH compartment may be concerned with packaging of these molecules, just as it seems to be for lysosomal enzymes. Finally, both molecules may have an intracellular degradative path (analogous to that for collagen and other secretory products: Table 1), whose action is suppressed by amines. This might also result in increased output via an unchanged normal transport mechanism.

Membrane shedding

Membrane shedding is an active process and may be induced experimentally (Scott & Maercklein, 1977). Sabatini *et al.* (1982) argue that shedding initiated by limited proteolysis of a membrane component may have been an evolutionary progenitor of the secretory pathway in which the shedding process was moved to the interior of the cell in a vesicle that could fuse with the plasma membrane subsequent to the limited proteolytic event, thus initiating release of soluble products to the exterior. Thus another possible site of stimulation of plasminogen activator and thromboplastin production just described may be the shedding process *per se*. There are indications that chloroquine enhances the shedding of certain viral proteins from cell surfaces (Moore *et al.*, 1983), but much further study of this general possibility is needed. Fig. 2 suggests some of the sites of action of amines that pertain to release of macromolecules from cells.

Summary and some interpretations

We have reviewed the evidence that amines accumulate in intracellular vesicles of low pH, such as lysosomes and endosomes. There is consequent elevation of intravesicular pH, and inhibition of receptor–ligand dissociation often results from this pH change. We have argued that the capacity for fusion of such vesicles is also reduced by the high pH. We suggest that the variety of effects of amines on membrane flow and macromolecular transport we describe are at least partly due to such reduced fusion (Figs. 1 and 2).

We propose that an internal low pH may facilitate heterologous vesicle–vesicle and vesicle–plasma membrane fusion. There is some evidence that clathrin can accelerate phospholipid vesicle fusion *in vitro* at low pH (Blumenthal *et al.*, 1983) but no direct evidence on the role of intravesicular pH. This idea is consistent not only with the preceding discussion, but also with the fact that the intracellular membrane-bound compartments least involved in fusion events (e.g. mitochondria) are of neutral or alkaline internal pH. Membrane fusion is certainly required for the formation of vesicles at the periphery of the Golgi apparatus, and possibly earlier in the transport and processing of biosynthetic products in the Golgi (Bergeron *et al.*, 1982). Thus the accumulation of amines in the

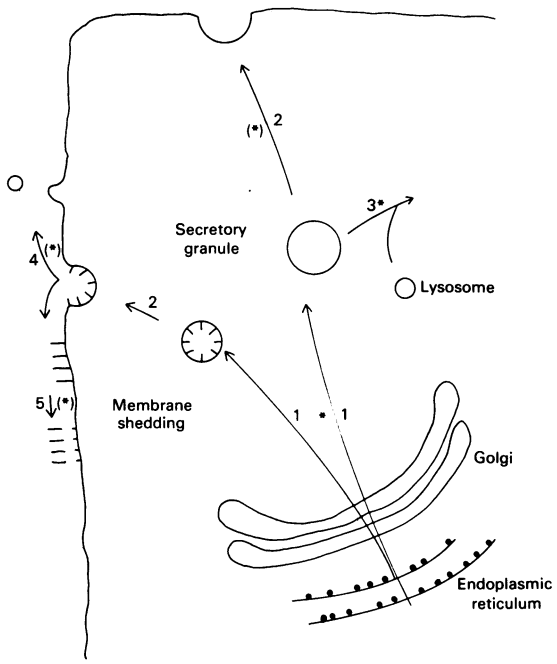

Fig. 2. *Effects of amines on exocytic pathways*
* Indicates points at which amines have been shown to act; (*) indicates points at which amines may have an effect, but for which the evidence is incomplete. Note that each arrow represents a segment of a pathway which may itself comprise several steps. Key: 1, synthesis and packaging of contents; 2, fusion with plasma membrane leading to exocytosis; 3, crinophagy; 4, shedding of membrane by outward vesiculation; 5, release of membrane components by selective proteolysis.

Golgi may be responsible for several effects on the flow of macromolecules along their translocation pathways.

The status of the plasma membrane in this view is complex. It might be argued that the pH dictating the fusion step in endocytosis is that of the extracellular fluid, in which case the inhibitory effects of amines on this process are not explained. However, the rapidity of acidification of the newly formed endocytic vesicles allows the possibility that plasma membrane invaginations might temporarily sequester areas which are of lower pH than that of the bulk extracellular fluid even before fusion, since the proton pumping enzyme(s) are probably present on the plasma membrane. Were this the case, then an acid pH could again be a factor determining membrane fusion at the plasma membrane. The inhibition of endocytosis by weak bases thus may again reflect elevation of pH in a sequestered compartment.

From the data on the dependence of response on the concentration of amines, we anticipate that most responses involving membrane flow will be biphasic, with inhibitory effects at low amine concentration, giving way to stimulatory ones at higher concentrations. We suggest that the reported dichotomy between different amines in intracellular membrane fusion systems (D'Arcy Hart, 1982) may result from this concentration dependence. Such dose–response data are lacking for most systems.

We have argued that the down-regulation of surface receptors induced by amines may result both from the inhibition of membrane fusion and the prevention of dissociation of internalized ligand–receptor complexes. Here we point out further that the recent awareness that plasma membrane transport sites are often regulated by reversible endocytosis (Lienhard, 1983) also suggests that the concentration of such sites may be similarly affected by amines. Some recent work on sodium channels is consistent with this view (Waechter *et al.*, 1983). That the effect of amines operates on many membrane proteins is indicated by the data of Schneider & Trouet (1981) who estimate that about 30% of surface antigens become unavailable during amine treatment of fibroblasts. Significant effects of amines on the turnover of plasma membrane proteins and other components can be anticipated in view both of their effects on membrane fusion *per se* and on receptor recycling.

Besides the effects of amines that involve changes in membrane fusion, it may be expected that a variety of proteolytic conversion events not yet studied will be inhibited by amines. Speculating more freely, one can envisage that modification of such proteolytic events by amines might have an impact on shedding pathways besides those wrought by amines on their membrane flow components. For instance, were the rate of release of shed proteins determined by the rate of limited cleavage at the cell surface, this might be controlled significantly by the local acidification at the site of forming endosomes. As argued already, amines might restrict this component of the shedding pathway. If, on the other hand, the secretion of a protein depends on how many molecules are cleaved from their membrane-binding, hydrophobic tails intracellularly, then amines could again restrict the secretory rate.

We suggest that a wider variety of secretory processes than so far studied are also susceptible to such influence, often in a biphasic manner. This may be indicated for example by the fact that some inducers of interferon output (notably tilorone: Thelmo & Levine, 1978) are amines.

The many effects of amines on membranes are of interest, and may ultimately seem not interpre-

tive problems, but rather important empirical and practical tools.

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